Express Mail No. **EV 346 811 873 US**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Zichria ZAKAY-RONES et al.

Confirmation No. 1732

Application No.: 10/800,256

Group Art Unit: 1614

Filing Date: March 11, 2004

Examiner:

Atty. Docket No.: 85189-5900

For: COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

SUBMISSION OF CERTIFIED PRIORITY DOCUMENT

Commissioner for Patents
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Sir:

Applicants have claimed priority of Israeli application no. IL145397 filed September 12, 2001, under 35 U.S.C. § 119. In support of this claim, a certified copy of said application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

Date: 10/8/04

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Enclosures

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1967–PATENT LAW -- 5727

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מספר: NUMBER	45397
תאריך Date	12-09-2001
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בקשה לפטנט
Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד – מקום ההתאגדות)
I (Name and address of applicant, and in case of body corporate-place of incorporation

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citizens)

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(בעברית) תכשירים ושיטות לטיפול בסרטן
(Hebrew)

(באנגלית) COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER
(English)

מבקש בזאת כי ינתן לי עליה פטנט
h pp patent to be granted to me in respect thereof.

*בקשה פטנט מוסף- Addition Application for Patent	* דרישה דין קדימה Priority Claim		
לבקשה/לפטנט to Patent/Application	מספר / סימן Number / Mark	תאריך Date	מדינת האגוד Convention Country
No.....מס			
dated.....מיום			
* יפוי כח: מיוחד – / עוד יוגש הוגש בענין			

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19/09/01

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תכשירים ושיטות לטיפול בסרטן

COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

COMPOSITIONS AND METHOD FOR TREATMENT OF CANCER

FIELD OF THE INVENTION

The present invention relates to treatment of cancer, specifically to the treatment of cancer with lentogenic viral strains and/or with viral proteins.

BACKGROUND OF THE INVENTION

Viruses are known to exert an oncolytic effect on malignant cells and the use of oncolytic viruses as therapeutic agents has been reported (Csatary et al. *Cancer Detect Prev* (1993) 17(6):619-27; Csatary et al. *Anticancer Research* (1999) 19(1B):635-8 and for review see Sinkovics *J. of Clinical Virology* (2000) 16: 1-15).

Oncolytic viruses, for example, the avian virus Newcastle disease virus (NDV), have been shown to be cytolytic to tumor cells in vivo and in vitro (Reichard et al. *J Surg Res* (1992) 52(5):448-53 and Bar Eli et al. *J Cancer Res Clin Oncol* (1996) 122: 1 – 7).

The Newcastle disease virus is an avian RNA paramyxovirus that causes Newcastle disease in different avian species (dependent on the virulence of the virus strain and on the age of the individual bird) but that is considered minimally pathogenic in humans. NDV is an enveloped virus containing a linear, non segmented, single-strand, negative sense RNA genome. The virion consists of a coiled nucleocapsid containing single stranded RNA and 6 structural polypeptides (30,000-75,000 M.W.). The nucleocapsides are coated with protein and lipid

envelopes. The glycoprotein complexe hemagglutinin-neuraminidase (HN) protrudes from the envelope allowing the virus to bind to cellular receptors. The fusion glycoprotein (F), which is also anchored in the viral membrane, is first expressed as an inactive precursor (F0), then cleaved post-translationally to produce two disulfide linked polypeptides (F1 and F2). The active F glycoprotein is involved in penetration of NDV into host cells by facilitating fusion of the viral envelope with the host plasma cell membrane. The matrix protein (M), is involved in viral assembly, and interacts with both the viral membrane and the nucleocapsid proteins. The state of proteolytic cleavage of the surface glycoproteins F and HN is responsible for the virulence of the different NDV strains.

All strains of NDV are morphologically indistinguishable. However, large differences exist in the virulence of different strains for chickens, eggs and tissue culture systems. These differences are expressed in the classification of the different strains as velogenic (highly pathogenic), mesogenic (intermediate in pathogenicity) and lentogenic (apathogenic) strains.

The HN and F surface glycoproteins of whole NDV are postulated to be involved in the effect of NDV on Daudi cells (M.Sc thesis by Alissa Waldman – Kegnovitch (1999) Dept. of Virology, Haddasa Medical School of the Hebrew University of Jerusalem).

The effect of oncolytic viruses on neoplastic cells is attributed to the enhancement of the sensitivity of the neoplastic cells to the cytolytic activity of tumor necrosis factors and to the immune stimulatory properties of these viruses.

NDV in animals induces locally chemokines and cytokines, such as tumor necrosis factor alpha, that effect T cell recruitment and activation (Schirrmacher et al. (1998) *Semin Oncol* 25(6):677-96 and Schirrmacher et al. (1999) *Int J Oncol* 14(2):205 – 15).There are other reports that attribute the killing effect of an attenuated strain of NDV (73-T) on neuroblastoma cells to direct cytolysis following replication of infectious virus (Lorence et al. *J.Nat.Cancer Inst.* (1994) 86(16) 1228-1233). On the other hand, the killing effect of a mesogenic strain of NDV (RO) on Daudi lymphoma cells and the effect of NDV Ulster strain on metastatic Esb lymphoma and B16-F10 melanoma was found to be unrelated to viral replication since UV inactivated viruses were found to be as effective as infectious viruses in killing these tumor cells (Tsadok-David et al. (1995) *J. Cancer Research Clinical Oncology* 121:169-174 and Schirrmacher et al. (1997) *Clin Cancer Res* 3(7):1135-48).

Present efforts at cancer therapy using viruses involve the use of live pathogenic viruses as cytolytic agents (see Csatary et al. above and US Patent Number 5,602,023 to Csatary). Alternative methods are mostly directed at developing vaccines for anti tumor immunization. For example, NDV is used in the preparation of an autologous tumor cell vaccine for humans (reviewed in Schirrmacher et al. (1998) *Semin Oncol* 25(6):677-96).

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for treatment of cancer that avoid contacting a patient with pathogenic strains of viruses. The compositions and methods of the invention utilize oncolytic properties of viruses and/or of viral proteins for the killing of neoplastic cells.

According to one embodiment of the invention the treatment for cancer utilizes at least one lentogenic strain of an oncolytic virus. Preferably, the virus used is a lentogenic (apathogenic) strain of Newcstle Disease Virus. More preferably, the HUJ strain of NDV (which is further described below) is utilized in the treatment of cancer.

According to a second embodiment of the invention the treatment for cancer utilizes at least one viral protein or subunit or analog thereof rather than whole viruses. The viral proteins utilized in this embodiment, which may be natural virus proteins or synthetic analogs thereof, are non infectious and can therefore be the product of any suitable strain of NDV; lentogenic, mesogenic or velogenic.

Thus, the compositions and methods of the invention provide a treatment for cancer that does not share the risks that may be involved in the use of live velogenic (highly pathogenic) or even mesogenic (intermediate in pathogenicity) strains of viruses.

The present invention provides a composition comprising at least the HUJ NDV strain. Preferably, the composition comprises 10^6-10^{12} egg infectious dose 50% (EID_{50})/ one treatment of HUJ NDV strain. In an alternative

embodiment the composition of the invention contains at least one surface glycoprotein of NDV or a subunit or analog thereof. Further, the composition may comprise any combination of viral proteins or subunits or analogs thereof or a combination of whole viruses and viral proteins or subunits or analogs thereof.

In one embodiment of the invention the composition includes at least the F glycoprotein of NDV. In another embodiment the composition contains the F glycoprotein and the hemagglutinin activity containing subunit of the HN glycoprotein of NDV. In yet a further embodiment the composition comprises the F glycoprotein and the HN glycoprotein of NDV. The surface glycoproteins may be obtained from any naturally occurring strain of NDV. Preferably, the glycoproteins are obtained from a velogenic or a mesogenic NDV strain, such as the Roakin/46 V log NJ (RO) strain from the American type collection. Also, the glycoproteins may be obtained from genetically or otherwise engineered virus strains. Furthermore, the glycoproteins may be obtained from an expression system comprised of a mammalian expression plasmid or a viral vector. Alternatively, synthetic peptides or recombinant viral proteins, such as HN or F, may be used in the present invention.

It should be appreciated by persons skilled in the art that the term "protein analog" includes peptides having the functionality of viral counterparts (i.e. fusion, hemagglutinin and neuraminidase activities etc.) and not necessarily having the same sequence, secondary or tertiary structure as the viral counterparts. Thus, truncated or altered proteins displaying the same activity as

the NDV surface glycoproteins, may be used in the composition and method of the present invention.

The composition may be in any form suitable for administration to a patient, such as a suspension, an emulsion, a spray etc. The compositions of the invention may be adapted for any suitable route of administration, including but not limited to intravenous, oral, buccal, intranasal, inhalation, topical application to a mucosal membrane or injection, including intradermal, intrathecal, intracisternal, intralesional or any other type of injection.

The method of the invention for treatment of cancer, according to an embodiment of the invention, includes the step of administering to a patient a therapeutically effective amount of at least HUJ NDV. The HUJ NDV may be administered to the patient through any suitable route, as described above. One particularly preferred embodiment utilizes injection of the composition or of HUJ NDV strain or at least one of its components directly into a tumor or adjacent to the tumor.

According to another embodiment of the invention the method of the invention for treatment of cancer, includes the step of administering to a patient (through any suitable route, as described above) a therapeutically effective amount of at least one surface glycoprotein of NDV or a subunit or analog thereof. The surface glycoprotein may include at least the F glycoprotein of NDV, the F glycoprotein and the hemagglutinin activity containing subunit of the HN glycoprotein of NDV or the F glycoprotein and the HN glycoprotein of NDV.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in which:

Figure 1 is a graph showing the results of a representative experiment of the cytotoxic effect of two NDV strains (HUJ and MTH) on Daudi cells in culture;

Figure 2 is a graph showing apoptosis of Daudi cells in culture following interaction with two NDV strains (HUJ and MTH);

Figures 3A and 3B depict graphs showing thermostability of hemagglutinin activity after treatment at 56°C, for the two NDV strains (HUJ and MTH) in two experiments;

Figure 4 is a picture of an SDS Polyacrylamid gel after electrophoresis of NDV virion proteins (strains HUJ and MTH);

Figure 5 is a picture of an agarose gel after electrophoresis of the DNA products of RT-PCR amplification of NDV MTH and HUJ RNAs;

Figures 6A and 6B show graphs of the results of incubation of Daudi cells with surface glycoproteins from different NDV strains (Roakin and B-1);

Figure 7 depicts a graph showing the inhibition of cellular DNA synthesis in response to incubation of NDV with Daudi cells (D-2);

Figure 8 depicts the chromium ^{56} release from NDV infected cells cells;

Figures 9A, B and C depict graphs showing the effect of NDV propogated in tissue culture or in embryonated eggs on Daudi cells: the effect on the total number of cells (Fig. 9A), percentage of dead cells following infection (Fig. 9B) and the effect of treatment with trypsin on the cytotoxic activity of the NDV (Fig. 9C); and

Figure 10 shows a histogram of the F glycoprotein activity as indicated by hemolysis of erythrocytes.

DETAILED DESCRIPTION OF THE INVENTION

A modified lentogenic NDV strain termed HUJ is reported and described below. According to an embodiment of the invention, HUJ is used, together with an appropriate carrier such as Human Serum Albumin (HSA) or any suitable adjuvant, in the preparation of a composition for the treatment of cancer. All types of cancers may be included in the scope of the present invention. As a non limiting example, the following cancers can be treated according to the present invention: glioblastoma, lung carcinoma, breast cancer, prostate, melanoma, leukemia and sarcomas.

Further, evidence of the oncolytic effect of viral proteins is presented below. In accordance with a further embodiment of the invention, viral proteins, preferably, the F and/or HN glycoproteins or functional analogs or subunits of these proteins, are used, as above in the preparation of a composition for the treatment of cancer. The glycoproteins can be used in a composition with an adjuvant such as alum hydroxide or other known pharmaceutical carriers such as human serum albumin. Also, genetically engineered proteins having the viral functions, such as fusion and hemagglutination are included in the scope of this invention.

Treatment of patients with cancer, in accordance with embodiments of the present invention, can be systemic, where the above compositions or even isolated whole viruses and/or isolated proteins are administered to the patient. The form of administration may be intravenous, oral, buccal, intranasal, inhalation, topical application to a mucosal membrane, or injection, including intradermal,

intrathecal, intracisternal, intralesional or any other type of injection. Preferably, lentogenic NDV viruses (such as the HUJ strain), or viral proteins as described above or compositions according to the invention, are administered locally and directly to a tumor or to its vicinity. Typically, the form of local administration is by injection, for example, intralesional injection.

HUJ strain of NDV

A Lentogenic variant strain originated from B-1 V-188 (originated from the Hitchner B1, strain obtained from the American Type Collection in 1971) (Tzadok-David Y., Metzkin-Eizenberg M., Zakay-Rones Z. (1995) *J. Cancer Res Oncol.. Clin.* 121:169-174) was termed the HUJ NDV strain. The virus was passaged regularly in the allantoic cavity of embryonated eggs, and thus modified (since 1982 about 50 passages were done from E4 stock). The HUJ strain was cloned from passage number 50 by end point dilution and characterization was carried out on the cloned strain. The cloned HUJ strain was compared to the cloned MTH-68 strain of NDV, which is an attenuated "H" strain obtained by serial passage through eggs (allantoic fluid), manufactured in Hungary by Phylasia-Sanofi. (Csatary et al. *Anticancer Research* (1999) 19(1B):635-8) Allantoic fluid containing virus and virus purified on sucrose gradients, were compared.

The cloning of the HUJ strain: Serial passages were carried out at limited dilutions in 10-11 day old chicken embryonated eggs. Allantoic fluid from the

higher dilution (in which only 1/6 eggs is virus positive) was collected and further passaged in serial dilutions as in the cloning procedure.

The HUJ virus was not identical to the non cloned strain described in Tzadok-David et al,. Cultivation, concentration and purification were carried out using routine methods (Tzadok-David et al, and Slosaris M., Levy B., Katz E., Levy R., Zakay-Rones Z. (1989) *Avian Dis.* 33:248-253).

Preparation of virus: From 750 incubated eggs about 640 embryonated eggs (10-11 days) were inoculated into the allantoic cavity with 10^5-10^6 embryo infectious dose 50% (EID50) /egg. Embryos dying within the first 24 hr were discarded. After 72 hr, eggs with live embryos only were chilled at 4° for 16-18 hr. The allantoic fluid (~3 liters) was collected and centrifuged for 20 min at 2,000 rpm to remove debris and the supernatant with hemagglutination titer of (HA) 640-1280/ml was saved.

Purification: The virus was concentrated by centrifugation from infected allantoic fluid at 18,000 rpm in a Sorvall (RC-5) centrifuge using a SS-34 rotor, for 60 min at 4°C. The concentrated virus (100 ml – containing 32,000 HA units) was then purified by centrifugation for 90 min rpm through a sucrose gradient (10-60%) with an ultra centrifuge in a SW-27 rotor. The bands containing virus were collected, pelleted in an SW-27 rotor for 60 min at 24,000 rpm, resuspended, and the purified virus suspension was passed through milipore filters, aliquoted in 0.5 ml and kept at -70°C until use.

It will be appreciated by persons skilled in the art that other methods of virus concentration and purification may be used for obtaining the results above.

Biological assay: The titer of the virus was determined and stocks were prepared and stored at –70°C.

Sterility tests: The viral suspension was tested for bacterial and mycoplasma presence and was found to be sterile.

Biological characterization (MTH compared with HUJ)

1) Neuraminidase activity

Table 1

NDV strain	Allantoic fluid		Purified NDV	
	HA*	NA*	HA*	NA*
MTH	1024	100	16,000	1,400
	1024	384		
HUJ	1024	300	32-64,000	<2,400
	1024	<1000		

*reciprocal titer (dilution)

Table 2

Comparison of neuraminadase activities of two NDV strains

dilution	MTH	HUJ
1:50	0.55*	1.2
1:100	0.37	0.55
1:200	0.15	0.41

*OD at A 450nm is correlated with neuraminadase enzyme activity

The neuraminadase activity is higher in the HUJ strain.

2) Fusion activity

Fusion activity as determined by chicken erythrocyte hemolysis,. Fusion activity was found to be similar (1:32 – 1:64) for the two NDV strains).

3) Cytotoxic (oncolytic) effect

Table 3A

Cytotoxic effect of NDV on Daudi cells in culture

A. Percent of dead cells

Hours	MTH	HUJ	Control uninfected cells
1	20.0*	17.8	6.4
24	68.6	36.3	8.3
48	74.4	46.9	13.1
72	82.6	63.4	17.9
96	86.6	71.7	28.2

Average of 3 experiments
*percent dead cells

A graphic presentation of the results is shown in Fig. 1

Table 3B

B. Apoptosis of Daudi cells following treatment by NDV

Hours	MTH		HUJ		Control uninfected cells	
	% dead cells	Apop.*	% dead cells	Apop.*	% dead cells	Apop.*
1	22.0	ND	24.3	ND	4.8	ND
5	60.7	0.202	50.0	0.371	3.0	0.892
24	80.4	0.027	62.1	0.182	2.8	0.791
48	82.0	0.028	65.8	0.180	7.0	0.739
72	85.4	0.029	69.8	0.102	7.9	0.724
96	92.7	0.028	85.9	0.045	29.3	0.580

*Apoptosis determined by MTT method. MTT is a color reaction expressed by OD indicating apoptosis of cells. The intensity of OD correlate directly with cell viability and inversely with % of dead cells. Higher OD indicates higher viability.

The MTH effect on cells is more rapid but end results are similar both in killing and apoptosis. Both viruses also arrest cell replication. The results are presented in Fig. 2.

The effectiveness of the HUJ strain in killing cells in culture was tested in the range of $20EID_{50}$/cell - $2000EID_{50}$/cell and was found to be effective in this range. Thus, treatment that includes locally administering HUJ NDV to a tumor (alone or as an active component in a composition) would preferably consist of estimating the number of cells in the tumor or estimating the size of the tumor and administering HUJ NDV strain in the range of $20EID_{50}$/cell - $2000EID_{50}$/cell, or an equivalent amount of surface glycoproteins, according to the invention. Systemic treatment of a patient would preferably consist of administering at least one dose of 10^6-10^{12} EID_{50} of HUJ NDV strain or an equivalent amount of surface glycoproteins, according to the invention.

4) Thermostability of hemagglutinin activity after treatment at 56°C

Table 4A

	HA titer of allantoic fluid		HA titer of purified fluid	
Time lapse (minutes)	MTH	HUJ	MTH	HUJ
0	1024	1024	1024	2048
2	256	8	256	0
5	128	0	32	0
10	32	0	8	0
15	8	0	0	0
20	0	0	0	0
25	0	0	0	0
30	0	0	0	0

Table 4B

	HA titer of allantoic fluid		HA titer of purified fluid	
Time lapse (minutes)	MTH	HUJ	MTH	HUJ
0	512	512	1024	2048
2	512	128	512	0
5	512	0	16	0
10	512	0	8	0
15	64	0	0	0
20	8	0	0	0

A graphic presentation of the results shown in Tables 4A and 4B (results of two different experiments) are shown in Figs. 3A and B, respectively.

The hemagglutinin of the MTH is more thermostable, while hemagglutination activity in MTH is maintained for about 10 min, in 50°C in the HUJ there is no activity already after 2 min in both allantoic fluid and purified virus.

5. Sensitivity to thermolabile β inhibitors in sera

NDV strains are known to be sensitive to the β inhibitors, non specific, inhibitors in normal sera. Assays with horse sera that contain β inhibitors indicated that the HUJ strain is less sensitive to inhibitors than the MTH strain.

Table 5

Titer of inhibitors*

HUJ			MTH			Horse sera
Non-treated	58°	RDE	Non-treated	58°	RDE	
10*	10	10	160	ND	ND	1
10	10	10	320	160	40	2
10	10	10	80	ND	ND	3
2560	2560	1280	1280	2560	1280	Rabbit immune
*reciprocal titer						

Pathogenicity of NDV strains

6. Mean death time

The mean death time (MDT) of embryos indicates the virulence of the virus. The MDT was determined by the inoculation of SPF chicken eggs with serial dilutions of the cloned viruses. Death of embryos was determined in the different dilutions and the MDT (the mean death time of embryos infected with the highest dilution causing 100% death) was determined. The MDT for both strains was about the same, >96 h, indicating attenuation of the strains. The cloning procedure may select for a unique virus variant, as for the MTH strain, the original Hungarian strain (from the original ampules) was more virulent than the cloned MTH variant. MDT was less than 65 hours, indicating that the strain was mesogenic.

7. Replication of virus in chicken fibroblast cultures with and without trypsin (Try)

Table 6

MTH Titer ($TCID_{50}$)				HUJ Titer ($TCID_{50}$)			
-Try		+Try		-Try		+Try	
CPE	HA	CPE	HA	CPE	HA	CPE	HA
$10^{7.5}$	$10^{8.5}$	$10^{8.5}$	$10^{8.5}$	$10^{2.6}$	$10^{2.5}$	$10^{8.5}$	10^{5}
$10^{8.5}$	$10^{5.5}$	$10^{8.5}$	$10^{8.0}$				
	$10^{9.5}$		$10^{9.5}$	$10^{3.0}$	$10^{3.0}$	$10^{8.5}$	10^{6}

Cloned virus with the same HA titer (1:200) was inoculated into CEF monolayer cultures. Replication was followed by observation of CPE and hemagglutination (HA) assay in the medium and the titer ($TCID_{50}$) was determined.

Replication of the virus without trypsin indicates elevated virulence. It may indicate that the amino acid residues at the trypsin cleavage sites of the surface glycoproteins are basic (arginine or lysin).

The MTH strain replicates to similar titers with or without trypsin, unlike the HUJ strain that replicates to a much higher level in the presence of trypsin ($10^{3.0} \rightarrow 10^{8.5}{}_{TCID50}$). This would clearly indicate that the MTH strain is more pathogenic than the HUJ virus.

8. Serology

Most of the NDV strains are similar serologically. When using polyclonal rabbit anti NDV hyperimmune sera, both strains were similarly inhibited (HI titer 1:1280) (see table 1). However, human sera obtained from MTH-treated cancer patients had higher antibody titers to the homologus MTH strain than to

the HUJ strain, with respect to a number of biological activities. Also an immune rabbit serum that had similar HI antibody titer to both NDV strains, demonstrated different antibody titers to other viral activities.

Table 7

Inhibition of biological activities

Serum	MTH	HUJ
Inhibition of hemolysis*		
Treated patients		
1	40-80*	5-10
2	20-40	5-10
Rabbit (Hyper immune serum)	160	20
Hemagglutination inhibition*		
Treated patients		
1	2560	1280
2	2560	640
3	320	20
Rabbit (Hyper immune serum)	1280	1280
Neuraminadase inhibition*		
Treated patients		
1	1280	640
2	2560	320
3	320	80

*reciprocal titers

9. Neutralization in eggs

Sera from cancer patients treated with MTH was interacted with 100 EID_{50} of each of the two NDV strains. The mixture was then inoculated into 10-

11-day-old embryos. 48 hours later, neutralization of the virus was determined by hemagglutination assay.

Neutralizing serum titer was 1:320 against MTH and only 1:20 against HUJ (serum 1).

Neutralizing serum titer was 1:320 to MTH and only 1:80 for HUJ (serum 2).

Analysis of NDV HUJ strain proteins

In order to compare proteins of the two NDV strains MTH and HUJ, purified virion preparations (See NDV Preparation and Purification, above) were treated with SDS and the denatured proteins analyzed by electrophoresis in a 10% SDS Polyacrylamide gel. A picture of the SDS Polyacrylamide gel analysis of NDV virion protein is shown in Fig. 4. Electrophoresis in Polyacrylamide gel (10%) of the MTH and HUJ proteins was carried out with 2μg, 5μg, and 10μg viral proteins and the gel was subsequently stained with coommassie blue (Millar NS et al., (1988). *J. Gen. Virol.* 69 (3), 613-20). As observed in Fig. 4, six major proteins were resolved in the gel. These six proteins correspond to the known major structural proteins of NDV, P-69 kD; HN-74 kD; F0-62kD; F-56kD; NP – 60kD and M-38kD (Hightower, L.E., Morrison, T.B. and Bratt M.A. (1975) *J. Virol.* 16, 1599-1607). No differences in the apparent molecular weight of the major virion proteins of strains MTH and HUJ could be observed by this method.

RT-PCR analysis of NDV RNA

In order to compare the genomic RNA of the two NDV strains, MTH and HUJ, viral RNA was extracted and purified from the two virion preparations. For RT-PCR amplification, two oligonucleotide primers were designed, corresponding to the published sequence of the F gene of NDV La Sota strain (Millar NS et al., (1988). *J. Gen. Virol.* 69 (3), 613-20).

forward primer #29727, 5'CCGGAATTCACAGGCTGCCAGAATTTACA-3' (nucleotides 1120- 11220)
and backward primer #28749 CCATCGATGGTAGAAGAGTTTGGATCCC-3' (nucleotides 11150-11170).

The two genomic viral RNA preparations (1μg) were subjected to RT-PCR (AMV- RT at 43°C for 30 min followed by 25 cycles of PCR with TAQ polymerase (1min heat to 94°C, 30 sec at 65°C and 2 min at 72°C). The RT- PCR DNA products were resolved by electrophoresis on an agarose gel (1%), and stained with Ethidium Bromide. A picture of the agarose gel after electrophoresis of the DNA products of RT-PCR amplification of NDV MTH and HUJ RNAs is shown in Fig. 5, in which:

Lanes 1-2	MTH
Lanes 3-4	HUJ
Lane 5	MTH- withoutRT
Lane 6	HUJ- without RT
Lane 7	no viral RNA + RT

As seen in Fig. 5, (lanes 1 and 2) a DNA band of 1000 base pairs is observed in the amplification reaction of the MTH strain viral RNA, corresponding to the expected size of the F gene of NDV (Hightower, L.E.,

Morrison, T.B. and Bratt M.A. (1975) *J. Virol.* 16, 1599-1607). No DNA band could be observed after amplification of the HUJ strain genome using the same two PCR primers (lanes 3 and 4). This result indicated differences in the F- gene sequence of the two NDV strains.

NDV Surface Glycoproteins

Cytotoxicity of NDV surface glycoproteins

Adsorption of Newcastle Disease Virus surface glycoproteins to Daudi cells, without subsequent penetration, caused a rapid inhibition in cellular DNA synthesis, arrest in cell multiplication and eventually killing of the cells. Surface glycoproteins obtained from a mesogenic strain (Roakin) were more effective than those originating from a lentogenic strain (B-1).

Thus, it appeared that adsorbed glycoproteins distorted the integrity of the cell membrane, increasing its permeability, as was indicated by the elevation in ^{51}Cr release. The killing of the cells may presumably be linked to a specific cytopathic effect through signal transduction, mediated by the exogenous viral glycoproteins.

The strains used in these experiments are the lentogenic B-1 strain (B1) and the mesogenic Roakin /46 V log NJ strain (RO) obtained from the American type collection 1971.

Production of viral surface glycoproteins:

For the solubilization of hydrophobic membrane proteins, purified virus preparations were treated with a non ionic detergent NP-40 (Sigma), 0.2% for 30 min at 4°C. The detergent was extracted five times with a 1:1 volume of analytical ether (May and Baker Ltd., England). The ether was then evaporated by nitrogen. Viral core was removed by high-speed centrifugation (L-2 rotor Ti50) at 20,000 rpm for 45 min at 4°C. The surface glycoproteins in the supernatant were kept at –70°C. A buffer solution was subjected to an identical treatment and served for control purposes to assure that any effect would not be due to residual detergent.

It will be appreciated by persons skilled in the art that surface glycoproteins can be obtained by several other known methods and using other detergents.

Biological activities of NDV surface glycoproteins

The fraction obtained by treatment with NP-40 contained the surface glycoproteins Hemagglutinin-Neuraminadase (HN) and Fusion (F). In Table 8 (below) the biological properties of the glycoprotein fractions originating from a mesogenic (RHN) and a lentogenic (BHN) strain, are depicted. The infectivity of the two purified virus preparations from which the surface glycoproteins were extracted was $10^{9.3}$ EID50/0.2ml. No infectivity was recorded in the soluble fraction containing the surface glycoproteins Roakin and B-1 (RHN and BHN). Protein concentration (μg/ml) was similar in the two virus suspensions, before

extraction, a lower concentration was found, as expected, in the glycoproteins fraction.

Hemagglutination activity of the surface glycoproteins fraction was similar to the original whole virus preparation. Neuramindase activity, however, declined to 33 and 50% of the full value of intact virus suspension in Roakin and B-1 glycoproteins fractions, respectively. Hemolytic activity was high in the intact virus preparations while only a small portion of this activity (6%) was retained in the isolated surface glycoprotein fractions.

Table 8

Activities of intact virus and surface glycoprotein preparations.

Preparation	Infectivity* EID50/0.2ml	HA** $x10^3$	NA+	Hemolysis++	Proteins# µg/ml
RO	$10^{9.3}$	30	480	1.73	480
RHN	<1	29	160	0.09	165
B-1	$10^{9.3}$	39	640	1.81	470
BHN	<1	32	320	0.12	158

*Viral infectivity, calculated as median egg-infective dose/0.2 ml according to Reed and Muench.

**The reciprocal of the highest dilution that agglutinate CRBC
+The reciprocal of the highest dilution with enzyme activity (OD 540nm)
++Adsorbancy of the supernatant of CRBC treated with water (100% hemolysis) was measured at OD 540 nm.
#Determined by the Lowry method.

Interaction between surface glycoproteins and Daudi cells

Adsorption of RHN and B-HN virus to Daudi cells was demonstrated by positive immuno-fluorescent staining of live cells, 60 minutes after virus interaction. The number of the viable and dead Daudi cells after incubation with the different viral preparations was determined at different times (Figs. 6A and 6B). Cell multiplication was completely inhibited and after 72 hr all the cells were dead following interaction with whole virus preparations (RO, B-1), which were used as control and reference for the destructive capability of the surface glycoproteins. RHN fraction inhibited cell multiplication at a slower rate and over 70% of the cells were damaged and destroyed. B HN fraction, on the other hand, was ineffective on Daudi – 1 line and cell growth and the percentage of death were similar to control cells. When an additional line of Daudi cells was used (D-2) it exhibited a very high sensitivity, 100% of cells were killed by RHN fraction and 74% by the BHN fraction within 72 h (Figs. 6A and 6B). The subsequent experiments were carried out with the D-2 line.

DNA synthesis

A rapid inhibition of DNA synthesis (90-95%) was observed already following 1h of interaction of cells with NDV strains and fractions RO, RHN, B-1 and BHN. This inhibition was maintained throughout the experiment and reached 99% inhibition at 48h (the results are shown in Fig. 7 and in table 9 below).

Table 9

Inhibition of cellular DNA synthesis

Incubation (D-2)	%DNA inhibition NDV strain/fraction			
(hours)	RO	RHN	B-1	BHN
1	95	94.9	92.9	94
4	95.9	94.7	95.2	96.2
24	98.3	96.2	98.9	97.9
48	98.6	99	98.9	99

The inhibitory effect is NDV virus specific, as pretreatment of viral preparations (intact virus or isolated surface glycoproteins) with specific antiserum abolished cytotoxicity.

Elevation in cell membrane permeability

Cells were labeled with ^{51}Cr and interacted with the different NDV preparations. Following different time intervals radioactive leakage was determined in comparison with spontaneous release from uninfected control Daudi cells (see Fig. 8). A significant ^{51}Cr release was already observed 90 minutes following interaction with NDV RO (59%) and B-1 (79%), while only a low percentage of release was caused by RHN (12%) and BHN (6%). The release was elevated further to 6, 85, 23, and 18% at 4h post interaction with RO, B-1, RHN and BHN, respectively. At 24 h a total release (100%) resulted from the interaction with the intact virions, 65% release was recorded as a result of interaction with RHN and only 36% release was found in cells interacted with

BHN. In cells interacting with control fluids, or in uninfected cells, no elevation in membrane permeability and no cell damages was observed.

Tissue culture

Effect of virus cultivated in cultured primary chicken fibroblasts (CF)

The cytotoxic effect of NDV strains on Burkitt lymphoma Daudi cells was studied. Interaction of mesogenic (Roakin), as well as of active attenuated lentogenic strain (B-1) cultivated in the allantoic sac of embryonated eggs, lead to cell death (90%). However, lentogenic strains cultivated in chicken fibroblasts (CF) exhibited a very low activity with only 10% cell death (Figs. 9A-C). The activity was found to be dependent on the cleavage of the viral surface glycoproteins (Hemagglutinin Neuraminadase (HN) and Fusion (F)). While the glycoproteins of both the mesogenic and the lentogenic strains undergo cleavage by the proteases in the embryonated eggs, the lentogenic strain that has one glutamine residue in the cleavage site, is insensitive to the proteases of the CF. Cultivation of the virus in CF, in the presence of trypsin (CFT), or treatment of the purified virus preparation with trypsin (NDVT) restored virus activity (66% and 93% cell death, respectively). Neuraminidase and hemagglutinin activities are similar in treated and non-treated virus preparation as demonstrated by a hemagglutination test, viral adsorption on cells using fluorescent staining and a neuraminidase assay. The fusion glycoprotein of the CF grown virus is almost completely inactive, as indicated by lack of hemolysis of red blood cells (in 1:2 dilution only 31% hemolysis was recorded in comparison to 71% hemolysis in

1:32 dilution of egg grown virus). Trypsin elevated activity to 58% and 64% hemolysis in 1:16 dilution of CFT and NDVT, respectively (Table 10 and Fig. 10).

It seems, therefore, that the fusion glycoprotein which is responsible for the fusion of cell virus membranes plays a crucial role in the cytotoxic effect of the virus.

Table 10

Activity of F glycoproteins

virus	dilution	% hemolysis	dilution	% hemolysis	dilution	% hemolysis
Purified egg	(1:2)	95.4	(1:8)	89.4	(1:16)	71
CF	(1:2)	0.3				
CF+trypsin	(1:2)	80			(1:16)	58
In vitro Cultivated in CF+trypsin	(1:2)	88.8			(1:16)	64

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein above. Rather the scope of the invention is defined by the claims which follow.

CLAIMS

1. A composition for the treatment of cancer comprising the HUJ strain of NDV and a suitable carrier.

2. The composition according to claim 2 comprising $10^6 - 10^{12}$ EID_{50} / dose.

3. The composition according to claim 1 further comprising at least one viral glycoprotein.

4. The composition according to claim 3 wherein the at least one viral glycoprotein is from NDV.

5. The composition according to claim 4 wherein the at least one viral glycoprotein is the F glycoprotein of NDV.

6. The composition according to claim 4 wherein the at least one viral glycoprotein is the HN glycoprotein of NDV.

7. The composition according to claim 1 further comprising the F glycoprotein and HN glycoprotein of NDV.

8. The composition according to claims 4-7 wherein the NDV is a mesogenic strain.

9. A composition for the treatment of cancer comprising at least one viral glycoprotein or a subunit or analog thereof and a suitable carrier.

10. The composition according to claim 9 wherein the at least one viral glycoprotein is from NDV.

11. The composition according to claim 10 wherein the at least one viral glycoprotein is the F glycoprotein of NDV.

12. The composition according to claim 10 wherein the at least one viral glycoprotein is the HN glycoprotein of NDV.

13. The composition according to claim 9 further comprising NDV.

14. The composition according to claim 13 wherein the NDV is the HUJ strain.

15. An NDV virus of the HUJ strain useful in treating cancer.

16. A method for treating cancer in a patient comprising administering to the patient a composition according to any of claims 1 – 14.

17. The method according to claim 16 wherein the step of administering comprises locally administering the composition to a tumor or to its vicinity.

18. The method according to claim 17 wherein the step of administering comprises administering HUJ NDV in a range of $20EID_{50}$/cell - $2000EID_{50}$/cell.

19. A method for treating cancer in a patient comprising administering to the patient the NDV virus according to claim 15.

20. The method according to claim 19 wherein the step of administering comprises locally administering the NDV virus to a tumor or to its vicinity.

21. The method according to claim 20 wherein the step of administering comprises administering the NDV virus in a range of $20EID_{50}$/cell - $2000EID_{50}$/cell.

22. Use of an NDV virus of the HUJ strain in the preparation of a treatment for cancer.

23. Use of a viral glycoprotein or a subunit or analog thereof in the preparation of a treatment for cancer.

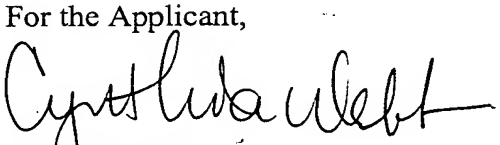
24. The use according to claim 23 wherein the viral glycoprotein is from NDV.

25. The use according to claim 24 wherein the viral glycoprotein is the F glycoprotein of NDV.

26. The use according to claim 24 wherein the viral glycoprotein is the HN glycoprotein of NDV.

27. The use according to claim 24 wherein the viral glycoprotein is a combination of the F glycoprotein of NDV and the HN glycoprotein of NDV.

For the Applicant,



Cynthia Webb
Webb, Ben-Ami & Associates

ABSTRACT

The present invention discloses lentogenic viral strains useful in the treatment of cancer. A preferred viral strain of Newcastle disease Virus (NDV) is specifically characterized in terms of biological activities. The present invention further discloses treatment of cancer by application of a new NDV strain to tumors. According to an alternative preferred embodiment the use of at least one viral protein or subunit or analog thereof is used in the treatment of cancer.

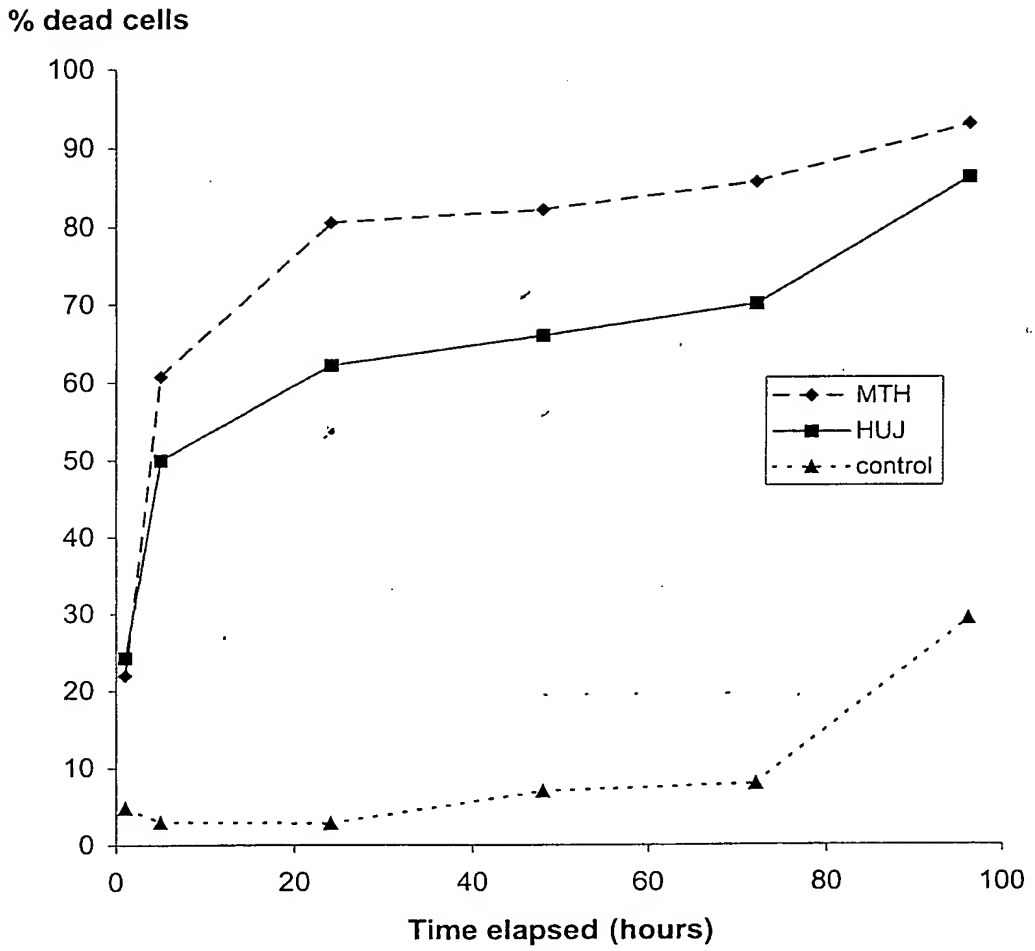

% dead cells
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
Time elapsed (hours)
MTH
HUJ
control

FIGURE 1

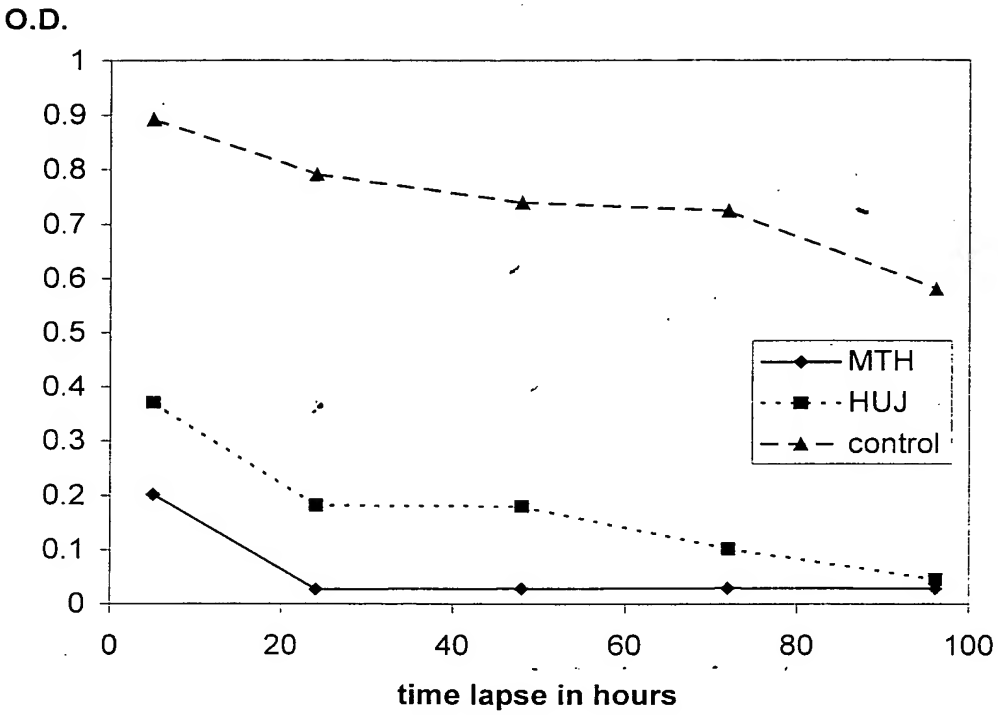

O.D.
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
time lapse in hours
MTH
HUJ
control

FIGURE 2

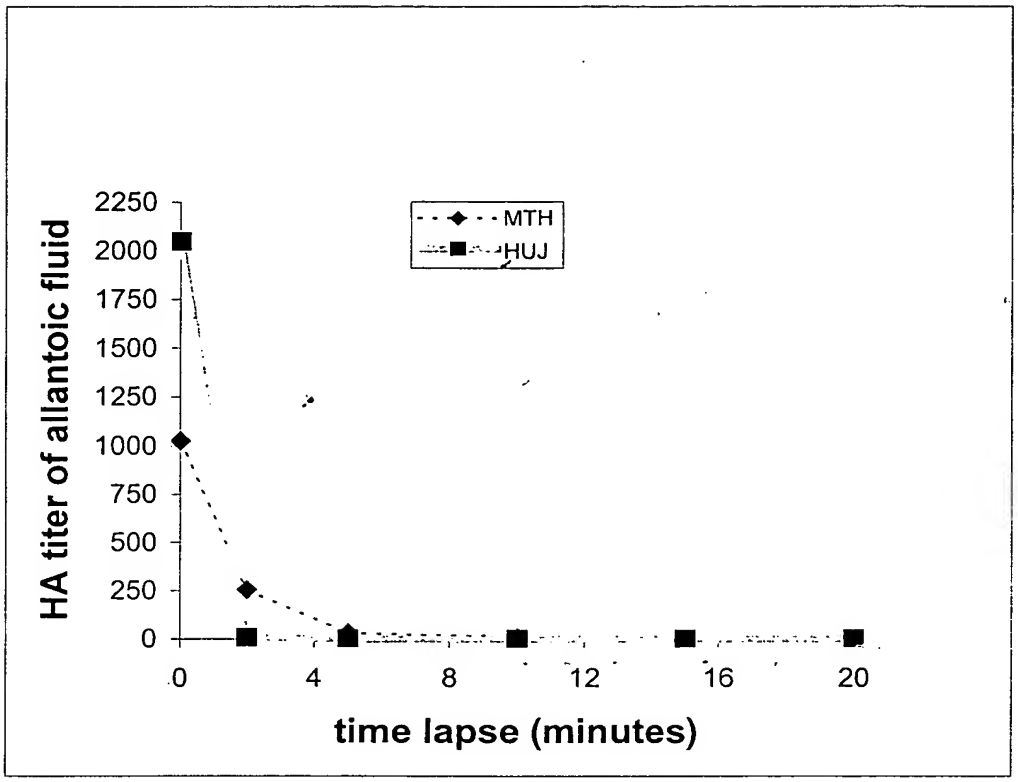

HA titer of allantoic fluid
2250
2000
1750
1500
1250
1000
750
500
250
0
0
4
8
12
16
20
time lapse (minutes)
MTH
HUJ

FIGURE 3A

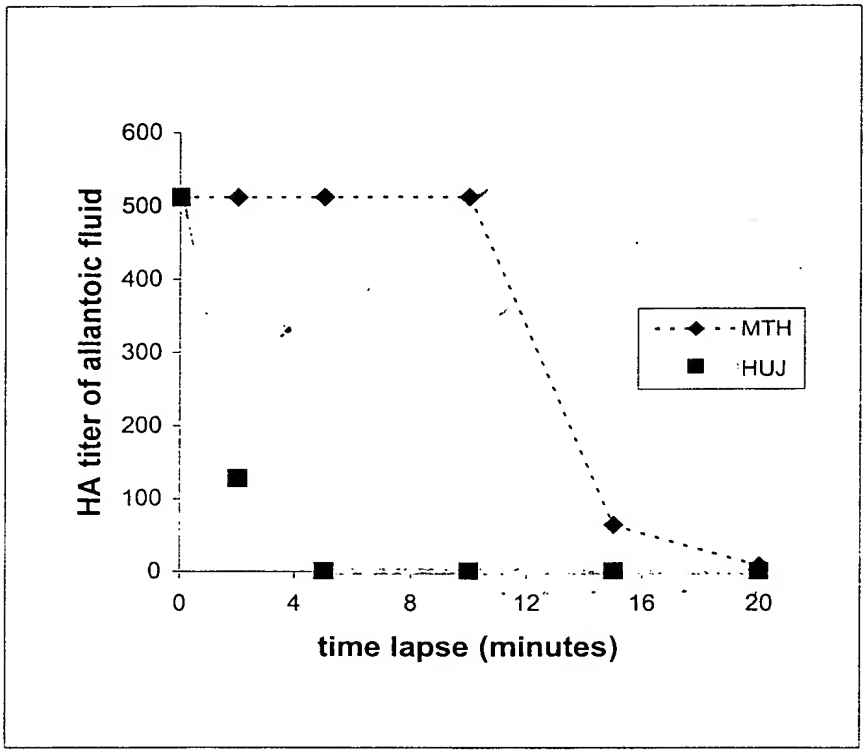

600
500
400
300
200
100
0
HA titer of allantoic fluid
0
4
8
12
16
20
time lapse (minutes)
MTH
HUJ

FIGURE 3B

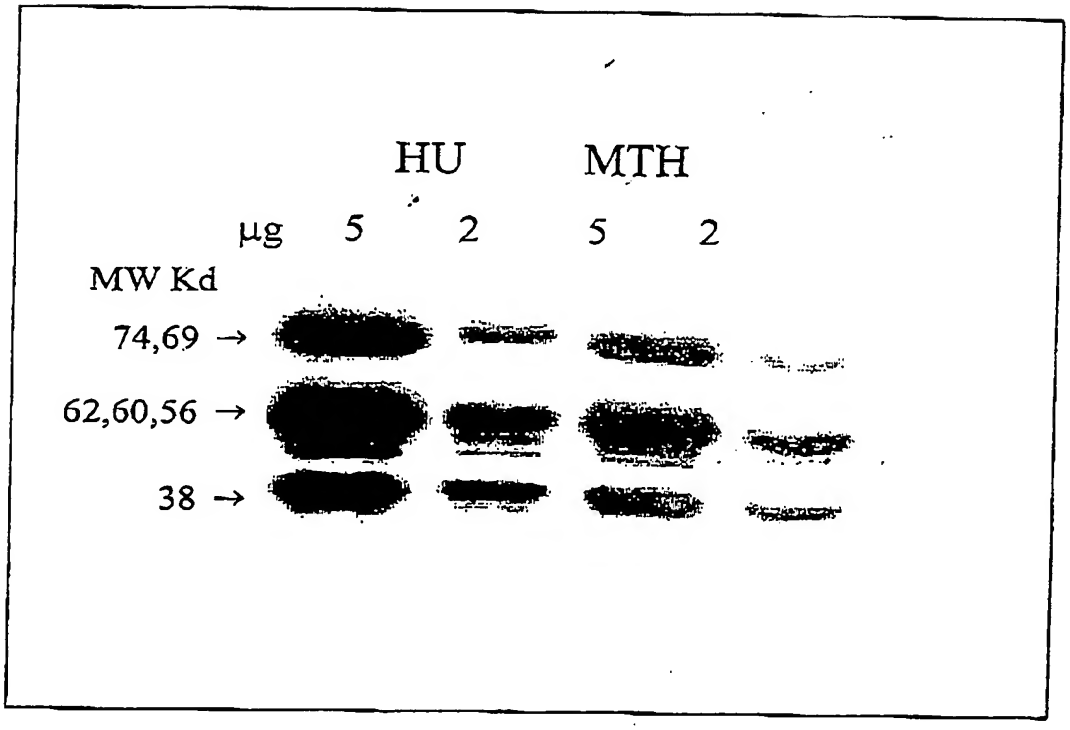


FIGURE 4

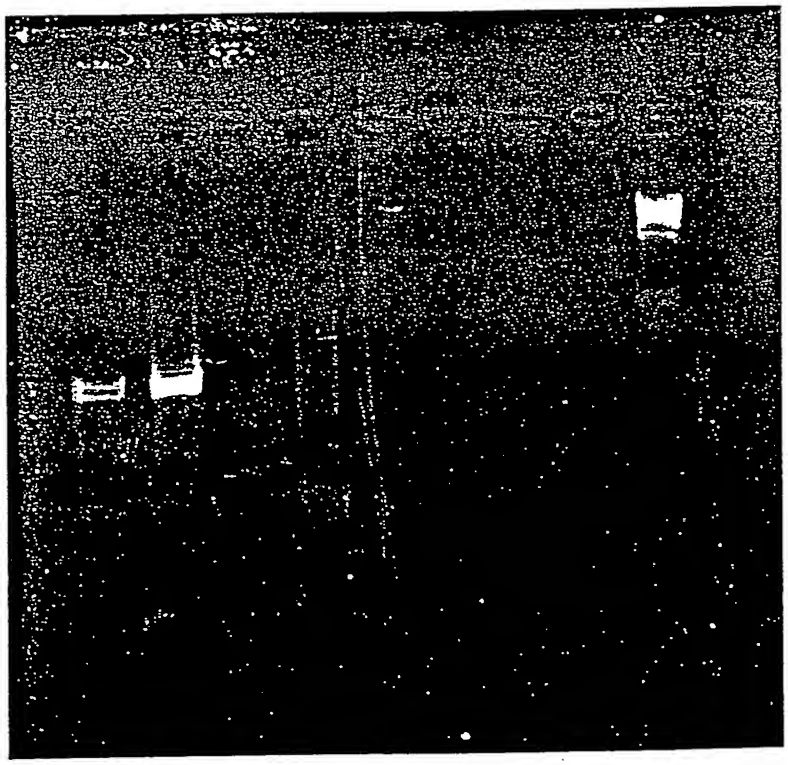

MTH-NDV
MTH-NDV
HUJ-NDV
HUJ-NDV
MTH-NDV w/o RT
HUJ-NDV w/o RT
No Virus + RT
λ Hind-III Marker

FIGURE 5

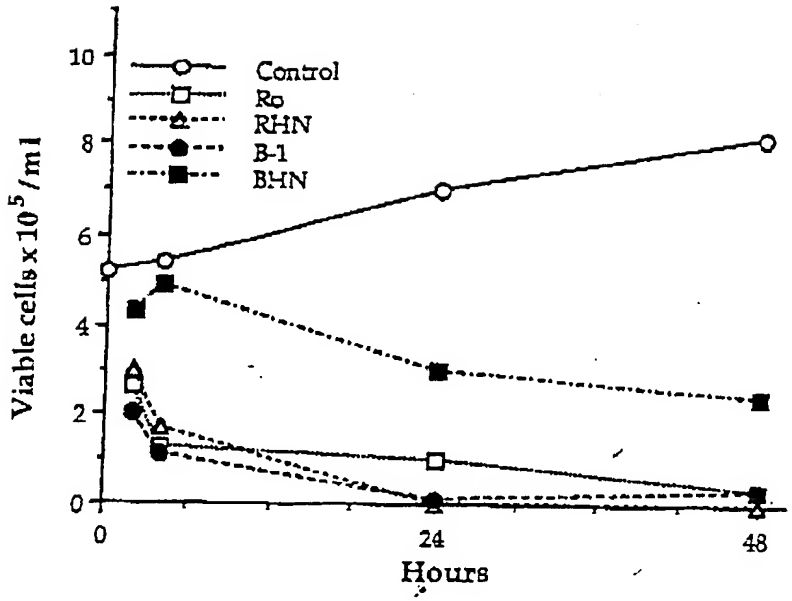


FIGURE 6A

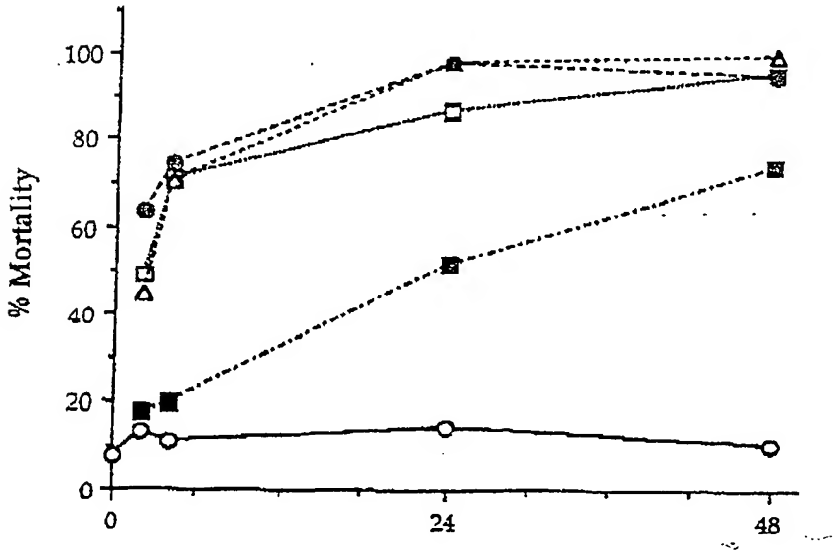


FIGURE 6B

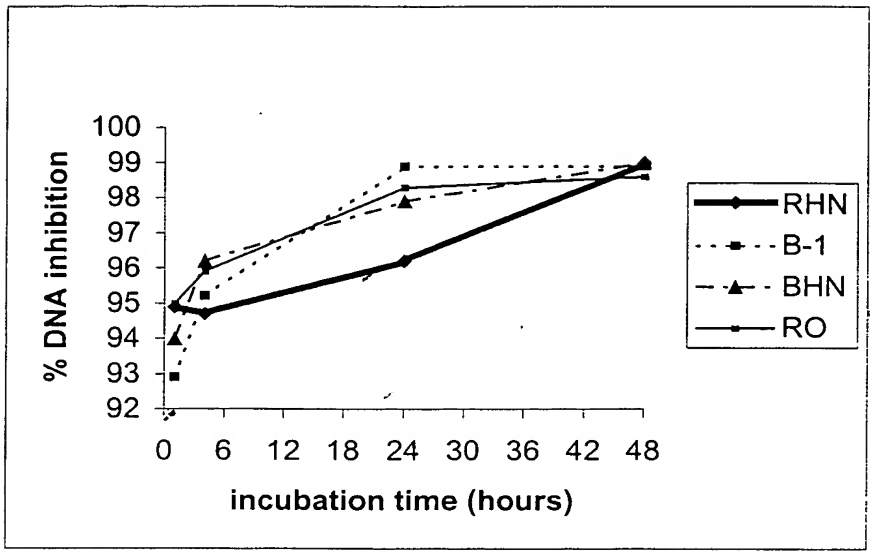
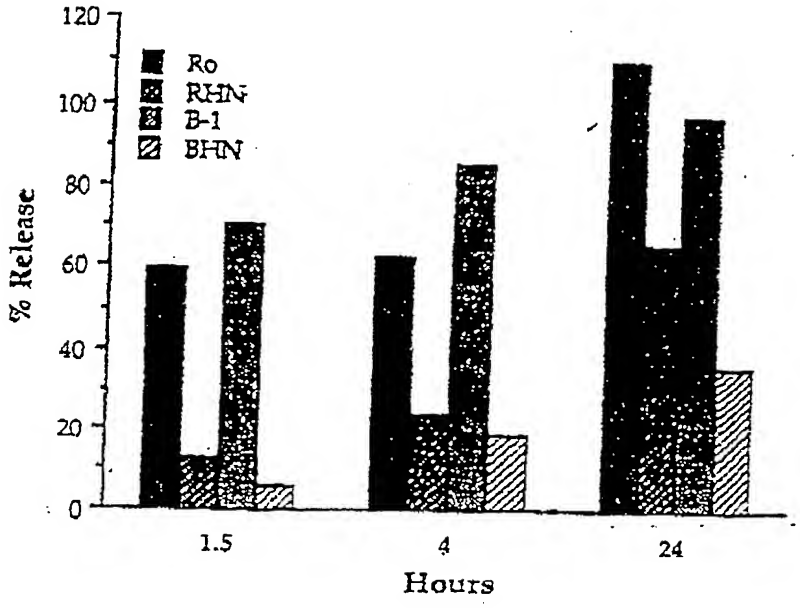

% DNA inhibition
100
99
98
97
96
95
94
93
92
0
6
12
18
24
30
36
42
48
incubation time (hours)
RHN
B-1
BHN
RO

FIGURE 7


120
100
80
60
40
20
0
% Release
Ro
RHN
B-1
BHN
1.5
4
24
Hours

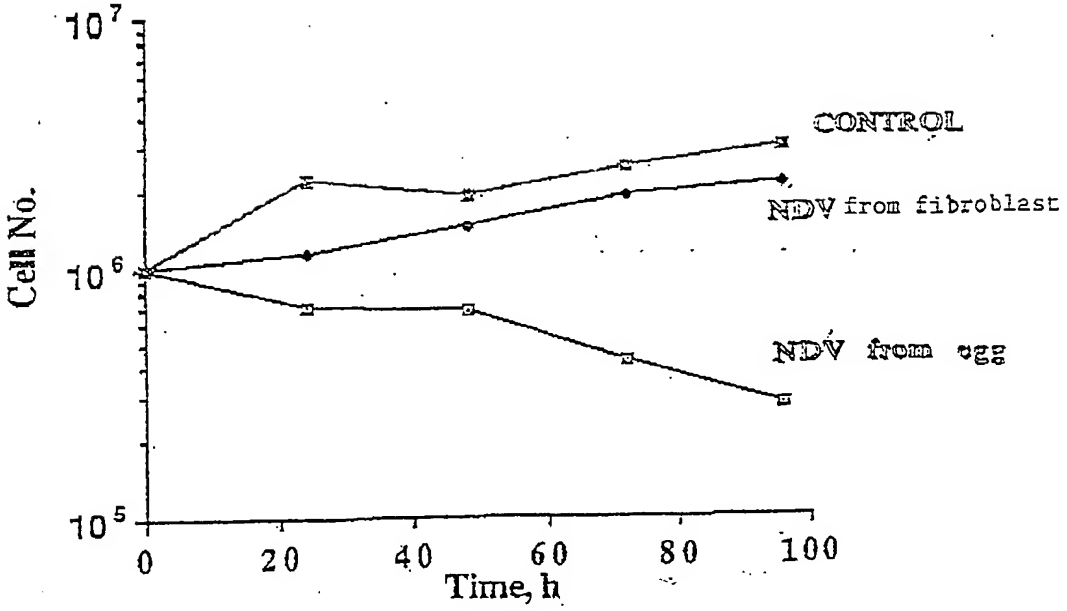

10^7
10^6
10^5
Cell No.
CONTROL
NDV from fibroblast
NDV from egg
0
20
40
60
80
100
Time, h

FIGURE 9A

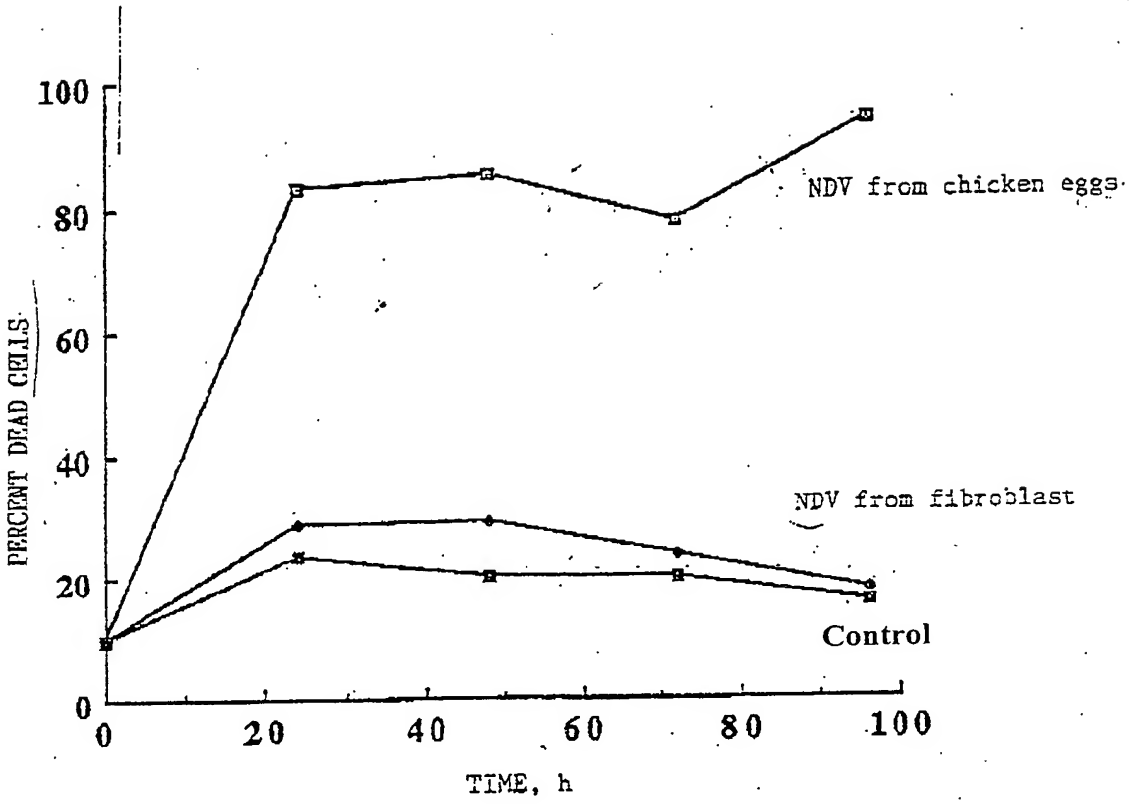

100
80
60
40
20
0
PERCENT DEAD CELLS
0
20
40
60
80
100
TIME, h
NDV from chicken eggs
NDV from fibroblast
Control

FIGURE 9B

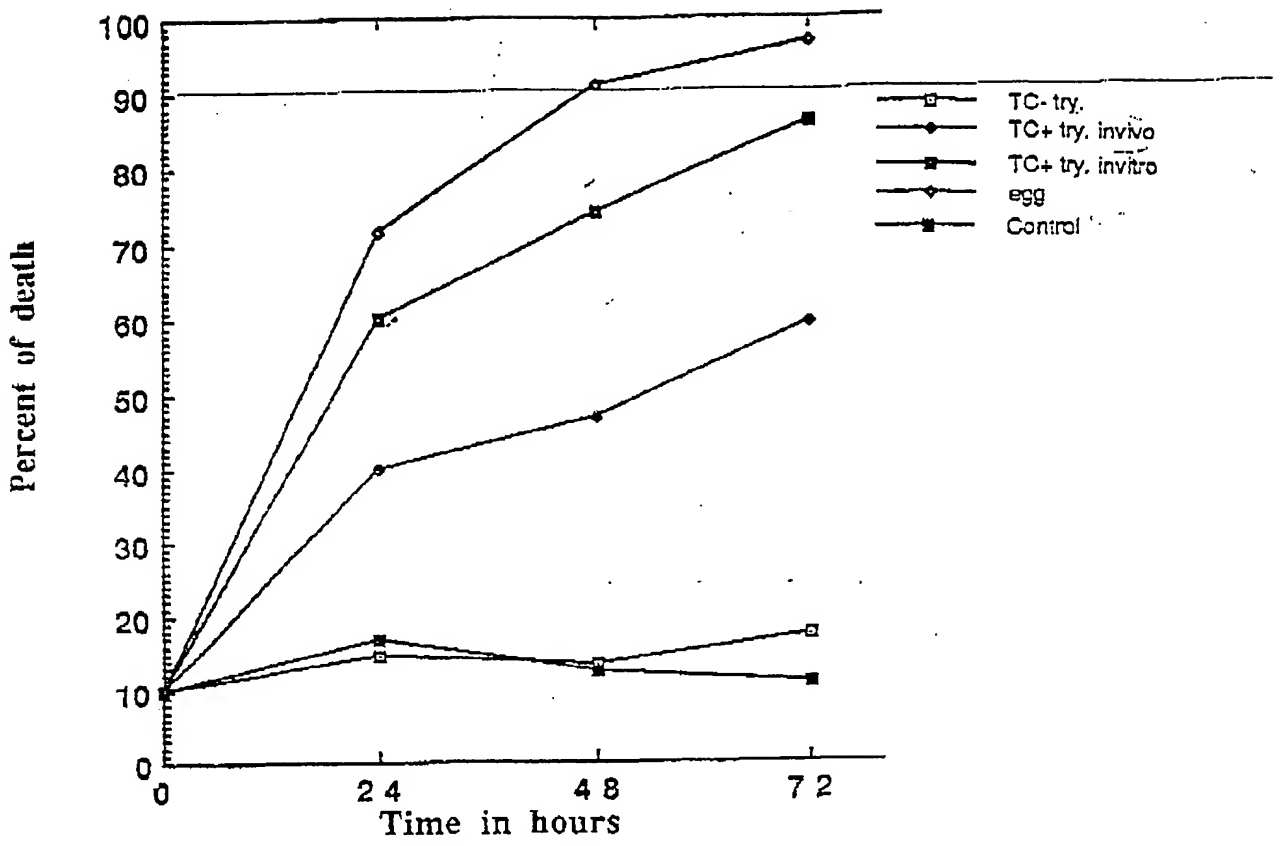
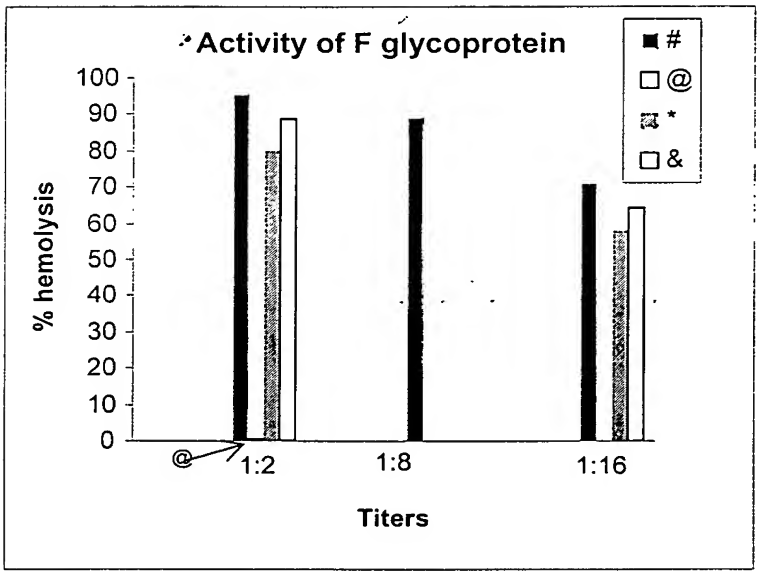

100
90
80
70
60
50
40
30
20
10
0
Percent of death
0
24
48
72
Time in hours
TC- try.
TC+ try. invivo
TC+ try. invitro
egg
Control

FIGURE 9C



(#) Purified egg virus
(@)TC virus
(*)TC virus + trypsin in vitro
(&)TC virus cultivated in the presence of trypsin

FIGURE 10